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(54) Title: GENETICALLY ENGINEERED VACCINE STRAIN

(57) Abstract

What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to knwon recombinant virus vaccines.

GENETICALLY ENGINEERED VACCINE STRAIN

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 07/713,967, filed June 11, 1991 which in turn is a continuation-in-part of application Serial No. 07/666,056, filed March 7, 1991, both of which are hereby incorporated herein by reference. Reference is also mad to copending U.S. applications Serial Nos. 715,921, filed Jun 14, 1991, 736,254, filed July 26, 1991, 776,867, filed October 22, 1991, and 820,077, filed January 13, 1992, all of which are hereby incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a modified poxvirus and to methods of making and using the same. More in particular, the invention relates to improved vectors for the insertion and expression of foreign genes for use as safe immunization vehicles to protect against a variety of pathogens.

Several publications are referenced in this application. Full citation to these references is found at the end of the specification immediately preceding the claims or where the publication is mentioned; and each of these publications is hereby incorporated herein by reference. These publications relate to the art to which this invention pertains.

BACKGROUND OF THE INVENTION

Vaccinia virus and more recently other poxviruses have been used for the insertion and expression of foreign gen s. The basic technique of inserting foreign genes into live infectious poxvirus involves recombination between pox DNA sequences flanking a foreign genetic element in a donor plasmid and homologous sequences present in the rescuing poxvirus (Piccini et al., 1987).

Specifically, the recombinant poxviruses ar constructed in two steps known in th art and analogous to the methods for creating synthetic recombinants of the vaccinia virus described in U.S. Patent Nos. 4,769,330, 4,772,848, and 4,603,112, and in copending application [illegible] the disclosures

regard reference is als made to copending U.S. applicati n Serial No. 537,890, filed June 14, 1990, als incorporated herein by reference.

First, the DNA gene sequence to be inserted into the virus, particularly an open reading frame from a non-pox source, is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA containing a nonessential locus. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1982).

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g. chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively gives a poxvirus modified by the presence, in a nonessential region of its genome, of foreign DNA sequences. The term "foreign" DNA designates exogenous DNA, particularly DNA from a non-pox source, that codes for gene products not ordinarily produced by the genome into which the exogenous DNA is placed.

Genetic recombination is in general the exchange of homologous sections of DNA between two strands of DNA. In certain viruses RNA may replace DNA. Homologous sections of nucleic acid are sections of nucleic acid (DNA or RNA) which have the same sequence of nucleotide bases.

Genetic recombination may take place naturally during the replication or manufacture of new viral genomes within the infected host cell. Thus, genetic recombination between viral genes may occur during the viral replication cycle that takes place in a host cell which is co-infected with two or more different viruses or other genetic constructs. A section of DNA from a first genome is used interchangeably in constructing the section of the genome of a second co-

inf cting virus in which the DNA is homologous with that of the first viral genome.

However, recombination can also take place between sections of DNA in different genomes that are not perfectly homologous. If one such section is from a first genome homologous with a section of another genome except for the presence within the first section of, for example, a gen tic marker or a gene coding for an antigenic determinant inserted into a portion of the homologous DNA, recombination can still take place and the products of that recombination are then detectable by the presence of that genetic marker or gene in the recombinant viral genome.

Successful expression of the inserted DNA genetic sequence by the modified infectious virus requires two conditions. First, the insertion must be into a nonessential region of the virus in order that the modified virus remain viable. The second condition for expressi n of inserted DNA is the presence of a promoter in the proper relationship to the inserted DNA. The promoter must be placed so that it is located upstream from the DNA sequence to be expressed.

Vaccinia virus has been used successfully to immunize against smallpox, culminating in the worldwide eradicati n of smallpox in 1980. In the course of its history, many strains of vaccinia have arisen. These different strains demonstrate varying immunogenicity and are implicated to varying degrees with potential complications, the most serious of which are post-vaccinial encephalitis and generalized vaccinia (Behbehani, 1983).

With the eradication of smallpox, a new role for vaccinia became important, that of a genetically engineered vector for the expression of foreign genes. Genes encoding a vast number of heterologous antigens have been expressed in vaccinia, often resulting in pr t ctive immunity against challenge by th corresponding pathogen (reviewed in Tartaglia et al., 1990a).

The genetic backgr und of the vaccinia v ctor has been shown t affect th protective efficacy of the expressed for ign immunogen. F r example, expression of Epstein Barr

Virus (EBV) gp340 in the Wyeth vaccine strain of vaccinia virus did not protect cott nt p tamarins against EBV virus induced lymphoma, while expression of the same gene in the WR laboratory strain of vaccinia virus was protective (Morgan et al., 1988).

A fine balance between the efficacy and the safety of a vaccinia virus-based recombinant vaccine candidate is extremely important. The recombinant virus must present the immunogen(s) in a manner that elicits a protective immune response in the vaccinated animal but lacks any significant pathogenic properties. Therefore attenuation of the vector strain would be a highly desirable advance over the current state of technology.

A number of vaccinia genes have been identified which are non-essential for growth of the virus in tissue culture and whose deletion or inactivation reduces virulence in a variety of animal systems.

The gene encoding the vaccinia virus thymidine kinase (TK) has been mapped (Hruby et al., 1982) and sequenced (Hruby et al., 1983; Weir et al., 1983). Inactivation or complete deletion of the thymidine kinase gene does not prevent growth of vaccinia virus in a wide variety of cells in tissue culture. TK^- vaccinia virus is also capable of replication *in vivo* at the site of inoculation in a variety of hosts by a variety of routes.

It has been shown for herpes simplex virus type 2 that intravaginal inoculation of guinea pigs with TK^- virus resulted in significantly lower virus titers in the spinal cord than did inoculation with TK^+ virus (Stanberry et al., 1985). It has been demonstrated that herpesvirus encoded TK activity *in vitro* was not important for virus growth in actively metabolizing cells, but was required for virus growth in quiescent cells (Jamieson et al., 1974).

Attenuation of TK^- vaccinia has been shown in mice in culated by the intracerebral and intraperitoneal routes (Buller et al., 1985). Attenuation was observed both for the WR neurovirulent laboratory strain and for the Wyeth vaccine strain. In mice inoculated by the intradermal route, TK^- recombinant vaccinia generated equivalent anti-

vaccinia neutralizing antibodies as compared with the parental TK^+ vaccinia virus, indicating that in this test system the loss of TK function does not significantly decrease immunogenicity of the vaccinia virus vector. Following intranasal inoculation of mice with TK^- and TK^+ recombinant vaccinia virus (WR strain), significantly less dissemination of virus to other locations, including the brain, has been found (Taylor et al., 1991a).

Another enzyme involved with nucleotide metabolism is ribonucleotide reductase. Loss of virally encoded ribonucleotide reductase activity in herpes simplex virus (HSV) by deletion of the gene encoding the large subunit was shown to have no effect on viral growth and DNA synthesis in dividing cells *in vitro*, but severely compromised the ability of the virus to grow on serum starved cells (Goldstein et al., 1988). Using a mouse model for acute HSV infection of the eye and reactivatable latent infection in the trigeminal ganglia, reduced virulence was demonstrated for HSV deleted of the large subunit of ribonucleotide reductase, compared to the virulence exhibited by wild type HSV (Jacobson et al., 1989).

Both the small (Slabaugh et al., 1988) and large (Schmitt et al., 1988) subunits of ribonucleotide reductase have been identified in vaccinia virus. Insertional inactivation of the large subunit of ribonucleotide reductase in the WR strain of vaccinia virus leads to attenuation of the virus as measured by intracranial inoculation of mice (Child et al., 1990).

The vaccinia virus hemagglutinin gene (HA) has been mapped and sequenced (Shida, 1986). The HA gene of vaccinia virus is nonessential for growth in tissue culture (Ichihashi et al., 1971). Inactivation of the HA gene of vaccinia virus results in reduced neurovirulence in rabbits inoculated by the intracranial route and smaller lesions in rabbits at the site of intradermal inoculation (Shida et al., 1988). The HA locus was used for the insertion of foreign genes in the WR strain (Shida et al., 1987), derivatives of the Lister strain (Shida et al., 1988) and the Copenhagen strain (Guo et al., 1989) of vaccinia virus.

Recombinant HA⁻ vaccinia virus expressing foreign genes have been shown to be immunogenic (Guo et al., 1989; Itamura et al., 1990; Shida et al., 1988; Shida et al., 1987) and protective against challenge by the relevant pathogen (Guo et al., 1989; Shida et al., 1987).

Cowpox virus (Brighton red strain) produces red (hemorrhagic) pocks on the chorioallantoic membrane of chicken eggs. Spontaneous deletions within the cowpox genome generate mutants which produce white pocks (Pickup et al., 1984). The hemorrhagic function (u) maps to a 38 kDa protein encoded by an early gene (Pickup et al., 1986). This gene, which has homology to serine protease inhibitors, has been shown to inhibit the host inflammatory response to cowpox virus (Palumbo et al., 1989) and is an inhibitor of blood coagulation.

The u gene is present in WR strain of vaccinia virus (Kotwal et al., 1989b). Mice inoculated with a WR vaccinia virus recombinant in which the u region has been inactivated by insertion of a foreign gene produce higher antibody levels to the foreign gene product compared to mice inoculated with a similar recombinant vaccinia virus in which the u gene is intact (Zhou et al., 1990). The u region is present in a defective nonfunctional form in Copenhagen strain of vaccinia virus (open reading frames B13 and B14 by the terminology reported in Goebel et al., 1990a,b).

Cowpox virus is localized in infected cells in cytoplasmic A type inclusion bodies (ATI) (Kato et al., 1959). The function of ATI is thought to be the protection of cowpox virus virions during dissemination from animal t animal (Bergoin et al., 1971). The ATI region of the cowp x genome encodes a 160 kDa protein which forms the matrix of the ATI bodies (Funahashi et al., 1988; Patel et al., 1987). Vaccinia virus, though containing a homologous region in its genome, generally does not produce ATI. In WR strain of vaccinia, the ATI region of the genome is translat d as a 94 kDa protein (Patel et al., 1988). In Copenhagen strain of vaccinia virus, most of the DNA sequences corresponding to the ATI regi n are deleted, with the remaining 3' end of the

region fused with sequences upstream from the ATI region to form open reading frame (ORF) A26L (Goebel et al., 1990a,b).

A variety of spontaneous (Altenburger et al., 1989; Drillien et al., 1981; Lai et al., 1989; Moss et al., 1981; Paez et al., 1985; Panicali et al., 1981) and engineered (Perkus et al., 1991; Perkus et al., 1989; Perkus et al., 1986) deletions have been reported near the left end of the vaccinia virus genome. A WR strain of vaccinia virus with a 10 kb spontaneous deletion (Moss et al., 1981; Panicali et al., 1981) was shown to be attenuated by intracranial inoculation in mice (Buller et al., 1985). This deletion was later shown to include 17 potential ORFs (Kotwal et al., 1988b). Specific genes within the deleted region include the virokine N1L and a 35 kDa protein (C3L, by the terminology reported in Goebel et al., 1990a,b). Insertional inactivation of N1L reduces virulence by intracranial inoculation for both normal and nude mice (Kotwal et al., 1989a). The 35 kDa protein is secreted like N1L into the medium of vaccinia virus infected cells. The protein contains homology to the family of complement control proteins, particularly the complement 4B binding protein (C4bp) (Kotwal et al., 1988a). Like the cellular C4bp, the vaccinia 35 kDa protein binds the fourth component of complement and inhibits the classical complement cascade (Kotwal et al., 1990). Thus the vaccinia 35 kDa protein appears to be involved in aiding the virus in evading host defense mechanisms.

The left end of the vaccinia genome includes two genes which have been identified as host range genes, K1L (Gillard et al., 1986) and C7L (Perkus et al., 1990). Deletion of both of these genes reduces the ability of vaccinia virus to grow on a variety of human cell lines (Perkus et al., 1990).

Fowlpox virus (FPV) is the prototypic virus of the Avipox genus of the Poxvirus family. The virus causes an economically important disease of poultry which has been well controlled since the 1920's by the use of live attenuated vaccines. Replication of the avipox viruses is limited to avian species (Matthews, 1982b) and there are no reports in the literature of the virus causing a productive

infection in any n n-avian species including man. This host restriction provides an inherent safety barrier to transmission of the virus to other species and makes us of FPV as a vaccine vector in poultry an attractive proposition.

FPV has been used advantageously as a vector expressing antigens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was expressed in an FPV recombinant (Taylor et al., 1988a). After inoculation of the recombinant into chickens and turkeys, an immune response was induced which was protective against either a homologous or a heterologous virulent influenza virus challenge (Taylor et al., 1988a). FPV recombinants expressing the surface glycoproteins of Newcastle Diseas Virus have also been developed (Taylor et al., 1990; Edbauer et al., 1990).

The use of live attenuated vectored vaccines present a number of potential advantages. The vaccines are inexpensive to produce and a number of poultry pathogens can potentially be incorporated into one vector. The immunogen is presented to the immune system in an authentic manner such that both humoral and cell mediated responses can be invoked. Because the disease agent is not replicating, side effects of vaccination are minimal and the continual re-introduction of the disease agent into the environment is eliminated.

It can be appreciated that provision of a novel vaccine strains having enhanced safety would be a highly desirable advance over the current state of technology. For instance, so as to provide safer vaccines or safer products from the expression of a gene or genes by a virus.

OBJECTS OF THE INVENTION

It is therefore an object of this invention to provide modified recombinant viruses, which viruses have enhanced safety, and to provide a method of making such recombinant viruses.

It is an additional object of this invention to provid a recombinant poxvirus vaccine having an increased level of safety compared to known recombinant poxvirus vaccin s.

It is a further object of this invention to provide a modified vector for expressing a gene product in a host, wherein the vector is modified so that it has attenuated virulence in the host.

It is another object of this invention to provide a method for expressing a gene product in a cell cultured *in vitro* using a modified recombinant virus or modified vector having an increased level of safety.

These and other objects and advantages of the present invention will become more readily apparent after consideration of the following.

STATEMENT OF THE INVENTION

In one aspect, the present invention relates to a modified recombinant virus having inactivated virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety. The functions can be non-essential, or associated with virulence. The virus is advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus and canarypox virus.

In another aspect, the present invention relates to a vaccine for inducing an immunological response in a host animal inoculated with the vaccine, said vaccine including a carrier and a modified recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety. The virus used in the vaccine according to the present invention is advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus and canarypox virus.

In yet another aspect, the present invention relates to an immunogenic composition containing a modified recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety. The modified recombinant virus includes, within a non-essential region of the virus genome, a heterologous DNA sequence which encodes an antigenic protein derived from a pathogen wherein the composition, when administered to a host, is capable of

inducing an immunological response specific to the protein encoded by the pathogen.

In a further aspect, the present invention relates to a method for expressing a gene product in a cell cultured *in vitro* by introducing into the cell a modified recombinant virus having attenuated virulence and enhanced safety.

In a still further aspect, the present invention relates to a modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the virus has attenuated virulence, and wherein the modified recombinant virus further contains DNA from a heterologous source in a nonessential region of the virus genome. In particular, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor or by utilizing naturally host restricted viruses. The virus used according to the present invention is advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus and canarypox virus. Advantageously, the open reading frame is selected from the group consisting of J2R, B13R + B14R, A26L, A56R, C7L - K1L, and I4L (by the terminology reported in Goebel et al., 1990a,b). In this respect, the open reading frame comprises a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range gene region or a large subunit, ribonucleotide reductase.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description, given by way of example, but not intended to limit the invention solely to the specific embodiments described, may best be understood in conjunction with the accompanying drawings, in which:

FIG. 1 schematically shows a method for the construction of plasmid pSD460 for deletion of thymidine kinase gene and generation of recombinant vaccinia virus vP410;

FIG. 2 schematically shows a method for the construction of plasmid pSD486 for deletion of hemorrhagic region and generation of recombinant vaccinia virus vP553;

FIG. 3 schematically shows a method for the construction of plasmid pMP494Δ for deletion of ATI region and generation of recombinant vaccinia virus vP618;

FIG. 4 schematically shows a method for the construction of plasmid pSD467 for deletion of hemagglutinin gene and generation of recombinant vaccinia virus vP723;

FIG. 5 schematically shows a method for the construction of plasmid pMPCSK1Δ for deletion of gene cluster [C7L - K1L] and generation of recombinant vaccinia virus vP804;

FIG. 6 schematically shows a method for the construction of plasmid pSD548 for deletion of large subunit, ribonucleotide reductase and generation of recombinant vaccinia virus vP866 (NYVAC);

FIG. 7 schematically shows a method for the construction of plasmid pRW842 for insertion of rabies glycoprotein G gene into the TK deletion locus and generation of recombinant vaccinia virus vP879;

FIG. 8 is a map of the EBV coding regions inserted into EBV Triple.1 plasmid;

FIG. 9 shows the DNA sequence (SEQ ID NO:213) of the synthetic spsAg gene and modified synthetic vaccinia virus H6 early/late promoter with the predicted amino acid sequence (SEQ ID NO:214);

FIG. 10 schematically shows a method for the construction of recombinant vaccinia virus vP856;

FIG. 11 shows the DNA sequence (SEQ ID NO:215) of the H promoter/lpsAg gene with the predicted amino acid sequence (SEQ ID NO:216);

FIG. 12 schematically shows a method for the construction of recombinant vaccinia virus vP896;

FIG. 13 shows the DNA sequence (SEQ ID NO:87) of the I3L promoter/S12/core gene with the predicted amino acid sequence (SEQ ID NO:217);

FIG. 14 schematically shows a method for the construction of recombinant vaccinia virus vP919;

FIG. 15 shows the DNA sequence (SEQ ID NO:218) of the EPV 42 kDa promoter/lpsAg gene with the predicted amino acid sequence (SEQ ID NO:219);

FIG. 16 shows the DNA sequence (SEQ ID NO:217) of a canarypox PvuII fragment containing the C5 ORF.

FIG. 17 schematically shows a method for the construction of recombinant canarypox virus vCP65 (ALVAC-RG);

FIG. 18 is a schematic of the JEV coding regions inserted in the vaccinia viruses vP555, vP825, vP908, vP923, vP857 and vP864;

FIG. 19 is a schematic of the YF coding regions inserted in the vaccinia viruses vP766, vP764, vP869, vP729 and vP725;

FIG 20 is a schematic of the DEN coding regions inserted in the vaccinia viruses vP867, vP962 and vP955;

FIG. 21 shows the nucleotide sequence (SEQ ID NO:221) of a 3661 base pair fragment of TROVAC DNA containing the F8 ORF;

FIG. 22 shows the DNA sequence (SEQ ID NO:222) of a 2356 base pair fragment of TROVAC DNA containing the F7 ORF;

FIG. 23 shows the nucleotide sequence of EIV HA (A1/Prague/56) (SEQ ID NO:279);

FIG. 24 shows the nucleotide sequence of EIV HA (A2/Fontainebleu/79) (SEQ ID NO:284);

FIG. 25 shows the nucleotide sequence of EIV HA (A2/Suffolk/89) (SEQ ID NO:300);

FIG. 26 shows the nucleotide sequence of FeLV-B Envelope Gene (SEQ ID NO:310);

FIG. 27 shows the nucleotide sequence of FeLV-A gag and partial pol genes (SEQ ID NO:324);

FIG. 28 shows the nucleotide sequence of the FHV-1 CO strain gD homolog gene (SEQ ID NO:290);

FIG. 29 shows the consensus F nucleotide sequence (mumps) represented by pURF3 (SEQ ID NO:370);

FIG. 30 shows the consensus HN nucleotide sequence (mumps) represented by pURHN5 (SEQ ID NO:371);

FIG. 31 shows the cytotoxic responses of spleen cells of mice and immunized with vaccinia virus or canarypox virus

vectors (NYVAC, ALVAC) or with vaccinia virus r canarypox virus recombinants expressing HIV III B env (vP911, vCP112);

FIG. 32 shows the sensitivity of the cytotoxic effector cells from the spleens of mice immunized with vCP112 to antibodies against cytotoxic T lymphocyte cell surface antigens Thy 1.2 and Lyt 2.2;

FIG. 33 shows the specificity of cytotoxic T lymphocyt antigen receptor for the HIV III B hypervariable V3 loop of gp120, but not for the V3 loop of HIV MN or SF2;

FIG. 34 shows the antibody responses to HIV III B gp120 of mice immunized with vectors (NYVAC, ALVAC) or with vaccinia virus recombinant vP911 or canarypox recombinant vCP112 expressing HIV-1 env (inverted triangle indicates time of administration of second inoculation);

FIG. 35 shows graph of rabies neutralizing antibody titers (RFFIT, IU/ml), booster effect of HDC and vCP65 ($10^{5.5}$ TCID50) in volunteers previously immunized with either the same or the alternate vaccine (vaccines given at days 0, 28 and 180, antibody titers measured at days 0, 7, 28, 35, 56, 173, 187 and 208);

FIG. 36 shows JEV cDNA sequences contained in vP908, vP555, vP923 and vP829;

FIG. 37 shows NEUT and HAI activities observed in swine immunized on days 0 and 28 with vP908, vP923, vP866 and PBS (arrows indicated days of inoculation);

FIG. 38 shows time course of viremia detected in individual pigs of each group immunized with PBS, vP866, vP908 or vP923 and then challenged with the B-2358/84 strain of JEV;

Fig 39 shows schematically the ORFs deleted to generate NYVAC;

DETAILED DESCRIPTION OF THE INVENTION

To develop a new vaccinia vaccine strain, NYVAC (vP866), the Copenhagen vaccine strain of vaccinia virus was modified by the deletion of six nonessential regions of the genome encoding known or potential virul nc factors. The sequential deletions are detailed below. All designations of vaccinia restriction fragments, open reading frames and

nucleotide positions are based on the terminology reported in Goebel et al., 1990a,b.

The deletion loci were also engineered as recipient loci for the insertion of foreign genes.

The regions deleted in NYVAC are listed below. Also listed are the abbreviations and open reading frame designations for the deleted regions (Goebel et al., 1990a,b) and the designation of the vaccinia recombinant (vP) containing all deletions through the deletion specified:

(1) thymidine kinase gene (TK; J2R) vP410;

(2) hemorrhagic region (u; B13R + B14R) vP553;

(3) A type inclusion body region (ATI; A26L) vP618;

(4) hemagglutinin gene (HA; A56R) vP723;

(5) host range gene region (C7L - K1L) vP804; and

(6) large subunit, ribonucleotide reductase (I4L) vP866 (NYVAC).

DNA Cloning and Synthesis. Plasmids were constructed, screened and grown by standard procedures (Maniatis et al., 1982; Perkus et al., 1985; Piccini et al., 1987). Restriction endonucleases were obtained from Bethesda Research Laboratories, Gaithersburg, MD, New England Biolabs, Beverly, MA; and Boehringer Mannheim Biochemicals, Indianapolis, IN. Klenow fragment of *E. coli* polymerase was obtained from Boehringer Mannheim Biochemicals. BAL-31 exonuclease and phage T4 DNA ligase were obtained from New England Biolabs. The reagents were used as specified by the various suppliers.

Synthetic oligodeoxyribonucleotides were prepared on a Biosearch 8750 or Applied Biosystems 380B DNA synthesizer as previously described (Perkus et al., 1989). DNA sequencing was performed by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase (Tabor et al., 1987) as previously described (Guo et al., 1989). DNA amplification by polymerase chain reaction (PCR) for sequence verification (Engelke et al., 1988) was performed using custom synthesized oligonucleotide primers and GeneAmp DNA amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, CT) in an automated Perkin Elmer Cetus DNA Thermal Cycler.

Excess DNA sequences were deleted from plasmids by restricti n endonucleas digesti n followed by limited digestion by BAL-31 exonuclease and mutagenesis (Mandecki, 1986) using synthetic oligonucleotides.

Cells, Virus, and Transfection. The origins and conditions of cultivation of the Copenhagen strain of vaccinia virus has been previously described (Guo et al., 1989). Generation of recombinant virus by recombination, *in situ* hybridization of nitrocellulose filters and screening for B-galactosidase activity are as previously described (Panicali et al., 1982; Perkus et al., 1989).

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

Example 1 - CONSTRUCTION OF PLASMID pSD460 FOR DELETION OF THYMIDINE KINASE GENE (J2R)

Referring now to FIG. 1, plasmid pSD406 contains vaccinia HindIII J (pos. 83359 - 88377) cloned into pUC8. pSD406 was cut with HindIII and PvuII, and the 1.7 kb fragment from the left side of HindIII J cloned into pUC8 cut with HindIII/SmaI, forming pSD447. pSD447 contains the entire gene for J2R (pos. 83855 - 84385). The initiation codon is contained within an NlaIII site and the termination codon is contained within an SspI site. Direction of transcription is indicated by an arrow in FIG. 1.

To obtain a left flanking arm, a 0.8 kb HindIII/EcoRI fragment was isolated from pSD447, then digested with NlaIII and a 0.5 kb HindIII/NlaIII fragment isolated. Annealed synthetic oligonucleotides MPSYN43/MPSYN44 (SEQ ID NO:1/SEQ ID NO:2)

```
                                    SmaI
MPSYN43  5'     TAATTAACTAGCTACCCGGG        3'
MPSYN44  3' GTACATTAATTGATCGATGGGCCCTTAA    5'
          NlaIII                    EcoRI
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were ligated with the 0.5 kb HindIII/NlaIII fragment into pUC18 vector plasmid cut with HindIII/EcoRI, generating plasmid pSD449.

To obtain a restricti n fragment containing a vaccinia right flanking arm and pUC vector sequences, pSD447 was cut with SspI (partial) within vaccinia sequences and HindIII at

All the recombinants containing SIV genes wer expressing th pertinent gen products. Th NYVAC recombinants vP873, vP943, vP948 and vP952 which contain the SIV env gen all expressed the authentic gp140. However, it is difficult to assess the processing of the gp140 protein to the 112 kDa and 28 kDa mature forms. No species with an apparent molecular weight of 140 kDa was precipitated by macaque anti-SIV sera from mock infected Vero cells, vP866 infected Vero cells and Vero cells infected with a NYVAC/SIV recombinant not containing the SIV env gene. Expression of the SIV gag encoded gene products by vP942, vp943, vp948, and vP952 was demonstrated using the pooled sera from macaques infected with SIV and the monoclonal antibody specific to the p28 gag component. Expression of the entir p55 gag protein without the pol region, which contains the protease function, by NYVAC (vP948) in Vero cells is evident. These results demonstrate that lack of SIV protease expression prevents the complete proteolysis of p55 into its mature form. This is demonstrated much more clearly when a monoclonal antibody specific to p28 was used to precipitate gag specific gene products from vP948 infected Vero cells. Contrary to this result, expression of SIV gag with the pol gene (includes protease) in vP943 infected Vero cells enabled the expressed p55 gag precursor polypeptide to be proteolytically cleaved to its mature forms.

Expression of both the p16 and p28 SIV gene products in vP942 and vP952 infected Vero cells was demonstrated using the pooled sera from macaques infected with SIV. Using th monoclonal antibody specific to p28 obviously only recognized the p28 expressed component.

Example 22 - CONSTRUCTION OF TROVAC RECOMBINANTS EXPRESSING THE HEMAGGLUTININ GLYCOPROTEINS OF AVIAN INFLUENZA VIRUSES

This Example describes the development of fowlpox virus recombinants expressing the hemagglutinin genes of three serotypes of avian influenza virus.

Cells and Viruses. Plasmids containing cDNA clones of the H4, H5 and H7 hemagglutinin genes were obtain d from Dr. Robert Webster, St. Jude Children's Research Hospital,

Memphis, Tennessee. The strain of FPV designated FP-1 has been described pr vi usly (Taylor et al., 1988a, b). It is an attenuated vaccine strain useful in vaccination of day old chickens. The parental virus strain Duvette was obtained in France as a fowlpox scab from a chicken. The virus was attenuated by approximately 50 serial passages in chicken embryonated eggs followed by 25 passages on chick embryo fibroblast (CEF) cells. This virus was obtained in September 1980 by Rhone Merieux, Lyon, France, and a master viral seed established. The virus was received by Virogenetics in September 1989, where it was subjected to four successive plaque purifications. One plaque isolate was further amplified in primary CEF cells and a stock virus, designated as TROVAC, was established. The stock virus used in the *in vitro* recombination test to produce TROVAC-AIH5 (vFP89) and TROVAC-AIH4 (vFP92) had been further amplified though 8 passages in primary CEF cells. The stock virus used to produce TROVAC-AIH7 (vFP100) had been further amplified through 12 passages in primary CEF cells.

Construction of Fowlpox Insertion Plasmid at F8 Locus. Plasmid pRW731.15 contains a 10 kbp PvuII-PvuII fragment cloned from TROVAC genomic DNA. The nucleotide sequence was determined on both strands for a 3661 bp PvuII-EcoRV fragment. This sequence is shown in FIG. 21. The limits of an open reading frame designated in this laboratory as F8 were determined within this sequence. The open reading frame is initiated at position 704 and terminates at position 1888. In order not to interfere with neighboring open reading frames, the deletion was made from position 781 to position 1928, as described below.

Plasmid pRW761 is a sub-clone of pRW731.15 containing a 2430 bp EcoRV-EcoRV fragment. The F8 ORF was entirely contained between an XbaI site and an SspI site in PRW761. In order to create an insertion plasmid which, on recombination with TROVAC genomic DNA would eliminate th F8 ORF, the following steps were followed. Plasmid pRW761 was completely digested with XbaI and partially digested with SspI. A 3700 bp XbaI-SspI band was isolated and ligated

with the annealed double-stranded oligonucleotides JCA017 (SEQ ID NO:191) and JCA018 (SEQ ID NO:192).

JCA017 (SEQ ID NO:191) 5' CTAGACACTTTATGTTTTTTAATATCCGGTCTT
3' AAAAGCTTCCCGGGGATCCTTATACGGGGAATAAT 3'

JCA018 (SEQ ID NO:192) 5' ATTATTCCCCGTATAAGGATCCCCCGGGAA
GCTTTTAAGACCGGATATTAAAAAACATAAAGTGT 3'

The plasmid resulting from this ligation was designated pJCA002. Plasmid pJCA004 contains a non-pertinent gene linked to the vaccinia virus H6 promoter in plasmid pJCA002. The sequence of the vaccinia virus H6 promoter has been previously described (Taylor et al., 1988a, b; Guo et al. 1989; Perkus et al., 1989). Plasmid pJCA004 was digest d with EcoRV and BamHI which deletes the non-pertinent gen and a portion of the 3' end of the H6 promoter. Annealed oligonucleotides RW178 (SEQ ID NO:193) and RW179 (SEQ ID NO:194) were cut with EcoRV and BamHI and inserted between the EcoRV and BamHI sites of JCA004 to form pRW846.

RW178 (SEQ ID NO:193): 5' TCATTATCGCGATATCCGTGTTAACTAGCTA
GCTAATTTTTATTCCCGGGATCCTTATCA 3'

RW179 (SEQ ID NO:194): 5' GTATAAGGATCCCGGGAATAAAAATTAGCT
AGCTAGTTAACACGGATATCGCGATAATGA 3'

Plasmid pRW846 therefore contains the H6 promoter 5' of EcoRV in the de-ORFed F8 locus. The HincII site 3' of the H6 promoter in pRW846 is followed by translation stop codons, a transcriptional stop sequence recognized by vaccinia virus early promoters (Yuen et al., 1987) and a SmaI site.

Construction of Fowlpox Insertion Plasmid at F7 Locus. The original F7 non-de-ORFed insertion plasmid, pRW731.13, contained a 5.5 kb FP genomic PvuII fragment in the PvuII site of pUC9. The insertion site was a unique HincII site within these sequences. The nucleotide sequence shown in FIG. 22 was determined for a 2356 bp region encompassing the unique HincII site. Analysis of this sequence revealed that the uniqu HincII site (FIG. 22, underlined) was situat d within an ORF encoding a polypeptide of 90 amino acids. The ORF begins with an ATG at position 1531 and terminates at position 898 (positions marked by arrows in FIG. 22).

The arms for the de-ORFed inserti n plasmid were derived by PCR using pRW731.13 as template. A 596 bp arm (designated as HB) corresponding to the region upstream from the ORF was amplified with oligonucleotides F73PH2 (SEQ ID NO:195) (5'-GACAATCTAAGTCCTATATTAGAC-3') and F73PB (SEQ ID NO:196) (5'-GGATTTTTAGGTAGACAC-3'). A 270 bp arm (designated as EH) corresponding to the region downstream from the ORF was amplified using oligonucleotides F75PE (SEQ ID NO:197) (5'-TCATCGTCTTCATCATCG-3') and F73PH1 (SEQ ID NO:198) (5'-GTCTTAAACTTATTGTAAGGGTATACCTG-3').

Fragment EH was digested with EcoRV to generate a 126 bp fragment. The EcoRV site is at the 3'-end and the 5'-end was formed, by PCR, to contain the 3' end of a HincII site. This fragment was inserted into pBS-SK (Stratagene, La Jolla, CA) digested with HincII to form plasmid pF7D1. The sequence was confirmed by dideoxynucleotide sequence analysis. The plasmid pF7D1 was linearized with ApaI, blunt-ended using T4 DNA polymerase, and ligated to the 596 bp HB fragment. The resultant plasmid was designated as pF7D2. The entire sequence and orientation were confirmed by nucleotide sequence analysis.

The plasmid pF7D2 was digested with EcoRV and BglII to generate a 600 bp fragment. This fragment was inserted into pBS-SK that was digested with ApaI, blunt-ended with T4 DNA polymerase, and subsequently digested with BamHI. The resultant plasmid was designated as pF7D3. This plasmid contains an HB arm of 404 bp and a EH arm of 126 bp.

The plasmid pF7D3 was linearized with XhoI and blunt-ended with the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs. This linearized plasmid was ligated with annealed oligonucleotides F7MCSB (SEQ ID NO:199) (5'-AACGATTAGTTAGTTACTAAAAGCTTGCTGCAGCCCGGGTTT TTTATTAGTTTAGTTAGTC-3') and F7MCSA (SEQ ID NO:200) (5'-GACTAACTAACTAATAAAAAACCCGGGCTGCAGCAAGCTTTTTGTAACTAACTAA TCGTT-3'). This was performed to insert a multiple cloning region containing the restriction sites for HindIII, PstI and SmaI between the EH and HB arms. The resultant plasmid was designated as pF7D0.

Construction of Insertion Plasmid for the H4 Hemagglutinin at the F8 Locus. A cDNA copy encoding the avian influenza H4 derived from A/Ty/Min/833/80 was obtained from Dr. R. Webster in plasmid pTM4H833. The plasmid was digested with HindIII and NruI and blunt-ended using the Klenow fragment of DNA polymerase in the presence of dNTPs. The blunt-ended 2.5 kbp HindIII-NruI fragment containing the H4 coding region was inserted into the HincII site of pIBI25 (International Biotechnologies, Inc., New Haven, CT). The resulting plasmid pRW828 was partially cut with BanII, the linear product isolated and recut with HindIII. Plasmid pRW828 now with a 100 bp HindIII-BanII deletion was used as a vector for the synthetic oligonucleotides RW152 (SEQ ID NO:201) and RW153 (SEQ ID NO:202). These oligonucleotides represent the 3' portion of the H6 promoter from the EcoRV site and align the ATG of the promoter with the ATG of the H4 cDNA.

RW 152 (SEQ ID NO:201): 5' GCACGGAACAAAGCTTATCGCGATATCCGTTA
AGTTTGTATCGTAATGCTATCAATCACGATTCTGT
TCCTGCTCATAGCAGAGGGCTCATCTCAGAAT 3'

RW 153 (SEQ ID NO:202): 5' ATTCTGAGATGAGCCCTCTGCTATGAGCAGGA
ACAGAATCGTGATTGATAGCATTACGATACAAACT
TAACGGATATCGCGATAAGCTTTGTTCCGTGC 3'

The oligonucleotides were annealed, cut with BanII and HindIII and inserted into the HindIII-BanII deleted pRW828 vector described above. The resulting plasmid pRW844 was cut with EcoRV and DraI and the 1.7 kbp fragment containing the 3' H6 promoted H4 coding sequence was inserted between the EcoRV and HincII sites of pRW846 (described previously) forming plasmid pRW848. Plasmid pRW848 therefore contains the H4 coding sequence linked to the vaccinia virus H6 promoter in the de-ORFed F8 locus of fowlpox virus.

Construction of Insertion Plasmid for H5 Hemagglutinin at the F8 Locus. A cDNA clone of avian influenza H5 derived from A/Turkey/Ireland/1378/83 was received in plasmid pTH29 from Dr. R. Webster. Synthetic oligonucleotides RW10 (SEQ ID NO:203) through RW13 (SEQ ID NO:206) were designed to overlap the translation initiation codon of the previously described vaccinia virus H6 promoter with the ATG of the H5

gene. The sequence continues through th 5' SalI site of the H5 gene and begins again at the 3' H5 DraI site containing the H5 stop codon.

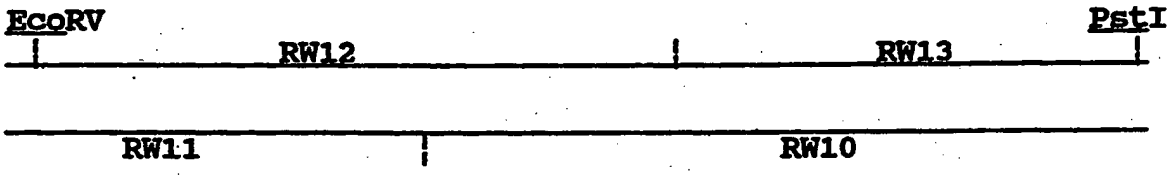
RW10 (SEQ ID NO:203): 5' GAAAAATTTAAAGTCGACCTGTTTTGTTGAGT TGTTTGCGTGGTAACCAATGCAAATCTGGTC ACT 3'

RW11 (SEQ ID NO:204): 5' TCTAGCAAGACTGACTATTGCAAAAAGAAGCA CTATTTCCTCCATTACGATACAAACTTAACG GAT 3'

RW12 (SEQ ID NO:205): 5' ATCCGTTAAGTTTGTATCGTAATGGAGGAAA TAGTGCTTCTTTTTGCAATAGTCAGTCTTGCTAGA AGTGACCAGATTTGCATTGGT 3'

RW13 (SEQ ID NO:206): 5' TACCACGCAAACAACTCAACAAAACAGGTCG ACTTTAAATTTTTCTGCA 3'

The oligonucleotides were annealed at 95°C for three minutes followed by slow cooling at room temperature. This results in the following double strand structure with the indicated ends.



Cloning of oligonucleotides between the EcoRV and PstI sites of pRW742B resulted in pRW744. Plasmid pRW742B contains the vaccinia virus H6 promoter linked to a non-pertinent gene inserted at the HincII site of pRW731.15 described previously. Digestion with PstI and EcoRV eliminates the non-pertinent gene and the 3'-end of the H6 promoter. Plasmid pRW744 now contains the 3' portion of the H6 promoter overlapping the ATG of avian influenza H5. The plasmid also contains the H5 sequence through the 5' SalI site and the 3' sequence from the H5 stop codon (containing a DraI site). Use of the DraI site removes the H5 3' non-coding end. The oligonucleotides add a transcription termination signal recognized by early vaccinia virus RNA polymerase (Yuen et al., 1987). To compl te the H6 promoted H5 construct, the H5 coding regi n was is lated as a 1.6 kpb SalI-DraI fragment from pTH29. Plasmid pRW744 was partially digested with DraI, the linear fragment isolated, recut with

SalI and the plasmid now with eight bases deleted between SalI and DraI was used as a vector for the 1.6 kpb pTH29 SalI and DraI fragment. The resulting plasmid pRW759 was cut with EcoRV and DraI. The 1.7 kbp PRW759 EcoRV-DraI fragment containing the 3' H6 promoter and the H5 gene was inserted between the EcoRV and HincII sites of pRW846 (previously described). The resulting plasmid pRW849 contains the H6 promoted avian influenza virus H5 gene in the de-ORFed F8 locus.

Construction of Insertion Vector for H7 Hemagglutinin at the F7 Locus. Plasmid pCVH71 containing the H7 hemagglutinin from A/CK/VIC/1/85 was received from Dr. R. Webster. An EcoRI-BamHI fragment containing the H7 gene was blunt-ended with the Klenow fragment of DNA polymerase and inserted into the HincII site of pIBI25 as PRW827. Synthetic oligonucleotides RW165 (SEQ ID NO:207) and RW166 (SEQ ID NO:208) were annealed, cut with HincII and StyI and inserted between the EcoRV and StyI sites of pRW827 to generate pRW845.

RW165 (SEQ ID NO:207): 5' GTACAGGTCGACAAGCTTCCCGGGTATCGCG
ATATCCGTTAAGTTTGTATCGTAATGAATACTCAA
ATTCTAATACTCACTCTTGTGGCAGCCATTCACAC
AAATGCAGACAAAATCTGCCTTGGACATCAT 3'

RW166 (SEQ ID NO:208): 5' ATGATGTCCAAGGCAGATTTTGTCTGCATTTG
TGTGAATGGCTGCCACAAGAGTGAGTATTAGAATT
TGAGTATTCATTACGATACAAACTTAACGGATATC
GCGATACCCGGGAAGCTTGTCGACCTGTAC 3'

Oligonucleotides RW165 (SEQ ID NO:207) and RW166 (SEQ ID NO:208) link the 3' portion of the H6 promoter to the H7 gene. The 3' non-coding end of the H7 gene was removed by isolating the linear product of an ApaLI digestion of pRW845, recutting it with EcoRI, isolating the largest fragment and annealing with synthetic oligonucleotides RW227 (SEQ ID NO:209) and RW228 (SEQ ID NO:210). The resulting plasmid was pRW854.

RW227 (SEQ ID NO:209): 5' ATAACATGCGGTGCACCATTTGTATAT
AAGTTAACGAATTCCAAGTCAAGC 3'

RW228 (SEQ ID NO:210): 5' GCTTGACTTGGAATTCGTTAACTTATA
TACAAATGGTGCACCGCATGTTAT 3'

The stop codon of H7 in PRW854 is f llowed by an HpaI site. The intermediate H6 prom ted H7 construct in the de-ORFed F7 locus (described, below) was generated by moving the pRW854 EcoRV-HpaI fragment into pRW858 which had been cut with EcoRV and blunt-ended at its PstI site. Plasmid pRW858 (described below) contains the H6 promoter in an F7 de-ORFed insertion plasmid.

The plasmid pRW858 was constructed by insertion of an 850 bp SmaI/HpaI fragment, containing the H6 promoter linked to a non-pertinent gene, into the SmaI site of pF7DO described previously. The non-pertinent sequences were excised by digestion of pRW858 with EcoRV (site 24 bp upstream of the 3'-end of the H6 promoter) and PstI. Th 3.5 kb resultant fragment was isolated and blunt-ended using the Klenow fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs. This blunt-ended fragment was ligated to a 1700 bp EcoRV/HpaI fragment derived from pRW854 (described previously). This EcoRV/HpaI fragment contains the entire AIV HA (H7) gene juxtaposed 3' to the 3'-most 24 bp of the VV H6 promoter. The resultant plasmid was designated pRW861.

The 126 bp EH arm (defined previously) was lengthened in pRW861 to increase the recombination frequency with genomic TROVAC DNA. To accomplish this, a 575 bp AccI/SnaBI fragment was derived from pRW 731.13 (defined previously). The fragment was isolated and inserted between the AccI and NaeI sites of pRW861. The resultant plasmid, containing an EH arm of 725 bp and a HB arm of 404 bp flanking the AIV H7 gene, was designated as pRW869. Plasmid pRW869 therefore consists of the H7 coding sequence linked at its 5' end to the vaccinia virus H6 promoter. The left flanking arm consists of 404 bp of TROVAC sequence and the right flanking arm of 725 bp of TROVAC sequence which directs insertion to the de-ORFed F7 locus.

Development of TROVAC-Avian Influenza Virus Recombinants. Insertion plasmids containing the avian influenza virus HA coding sequences were individually transfected into TROVAC infected primary CEF cells by using the calcium phosphate precipitation method previously

described (Panicali et al., 1982; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to HA specific radiolabelled probes and subjected to sequential rounds of plaque purification until a pure population was achieved. One representative plaque was then amplified to produce a stock virus. Plasmid pRW849 was used in an *in vitro* recombination test to produce recombinant TROVAC-AIH5 (vFP89) expressing the H5 hemagglutinin. Plasmid pRW848 was used to produce recombinant TROVAC-AIH4 (vFP92) expressing the H4 hemagglutinin. Plasmid pRW869 was used to produce recombinant TROVAC-AIH7 (vFP100) expressing the H7 hemagglutinin.

Immunofluorescence. In influenza virus infected cells, the HA molecule is synthesized and glycosylated as a precursor molecule at the rough endoplasmic reticulum. During passage to the plasma membrane it undergoes extensive post-translational modification culminating in proteolytic cleavage into the disulphide linked HA_1 and HA_2 subunits and insertion into the host cell membrane where it is subsequently incorporated into mature viral envelopes. To determine whether the HA molecules produced in cells infected with the TROVAC-AIV recombinant viruses were expressed on the cell surface, immunofluorescence studies were performed. Indirect immunofluorescence was performed as described (Taylor et al., 1990). Surface expression of the H5 hemagglutinin in TROVAC-AIH5, H4 hemagglutinin in TROVAC-AIH4 and H7 hemagglutinin in TROVAC-AIH7 was confirmed by indirect immunofluorescence. Expression of the H5 hemagglutinin was detected using a pool of monoclonal antibodies specific for the H5HA. Expression of the H4HA was analyzed using a goat monospecific anti-H4 serum. Expression of the H7HA was analyzed using a H7 specific monoclonal antibody preparation.

Immunoprecipitation. It has been determined that the sequence at and around the cleavage site of the hemagglutinin molecule plays an important role in determining viral virulence since cleavage of the hemagglutinin polypeptide is necessary for virus particles to be infectious. The hemagglutinin proteins of the

virulent H5 and H7 viruses possess more than one basic amino acid at the carboxy terminus f HA1. It is thought that this allows cellular proteases which recognize a series of basic amino acids to cleave the hemagglutinin and allow the infectious virus to spread both *in vitro* and *in vivo*. The hemagglutinin molecules of H4 avirulent strains are not cleaved in tissue culture unless exogenous trypsin is added.

In order to determine that the hemagglutinin molecules expressed by the TROVAC recombinants were authentically processed, immunoprecipitation experiments were performed as described (Taylor et al., 1990) using the specific reagents described above.

Immunoprecipitation analysis of the H5 hemagglutinin expressed by TROVAC-AIH5 (vFP89) showed that the glycoprotein is evident as the two cleavage products HA_1 and HA_2 with approximate molecular weights of 44 and 23 kDa, respectively. No such proteins were precipitated from uninfected cells or cells infected with parental TROVAC. Similarly immunoprecipitation analysis of the hemagglutinin expressed by TROVAC-AIH7 (vFP100) showed specific precipitation of the HA_2 cleavage product. The HA_1 cleavage product was not recognized. No proteins were specifically precipitated from uninfected CEF cells or TROVAC infected CEF cells. In contrast, immunoprecipitation analysis of the expression product of TROVAC-AIH4 (vFP92) showed expression of only the precursor protein HA_0. This is in agreement with the lack of cleavage of the hemagglutinins of avirulent subtypes in tissue culture. No H4 specific proteins were detected in uninfected CEF cells or cells infected with TROVAC.

Example 23 - DEVELOPMENT OF A TRIPLE RECOMBINANT EXPRESSING THREE AVIAN INFLUENZA GENES

Plasmid Construction. Plasmid pRW849 has been discussed previously and contains the H6 promoted avian influenza H5 gene. This plasmid was used for the development of vFP89. Plasmid pRW861 was an intermediate plasmid, described previously used in the development of vFP100. The plasmid contains the H6 promoted avian influenza H7 gene. Plasmid pRW849 was digested with SmaI

and the resulting 1.9 kbp fragment from the 5' end of the H6 promoter through the H5 gene was inserted at th SmaI site of pRW861 to produce pRW865. In order to insert the H4 coding sequence, plasmid pRW848 was utilized. Plasmid pRW848 was used in the development of vFP92 and contains the H6 promoted H4 gene (previously described). Plasmid pRW848 was digested with SmaI and a 1.9 kbp fragment containing the H6 promoted H4 coding sequence was then inserted into pRW865 at the SmaI site 5' of the H6 promoted H5 sequence. The resulting plasmid pRW872 therefore contains the H4, H5 and H7 coding sequences in the F7 de-ORFed insertion plasmid.

In order to direct insertion of the genes to the de-ORFed F8 locus, pRW872 was partially digested with SmaI, the linear fragment isolated and recut with HindIII. The 5.7 kbp SmaI to HindIII pRW872 fragment containing all thre H6 promoted avian influenza genes was blunt-ended and inserted into pCEN100 which had been cut with HincII. Plasmid pCEN100 is a de-ORFed F8 insertion vector containing transcription and translation stop signals and multiple insertion sites. Plasmid pCEN100 was generated as described below. Synthetic oligonucleotides CE205 (SEQ ID NO:211) and CE206 (SEQ ID NO:212) were annealed, phosphorylated and inserted into the BamHI and HindIII sites of pJCA002 (previously described) to form pCE72. A BglII to EcoRI fragment from pCE72 was inserted into the BglII and EcoRI sites of pJCA021 to form pCEN100.

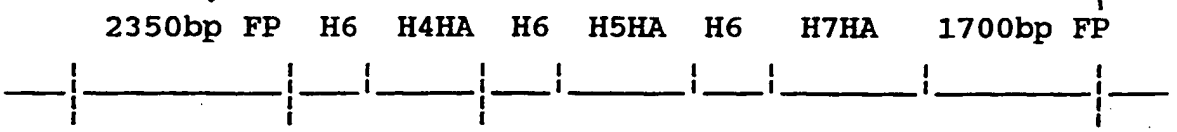
CE205 (SEQ ID NO:211): 5' GATCAGAAAAACTAGCTAGCTAGTACGTAGTT
AACGTCGACCTGCAGAAGCTTCTAGCTAGCTAGTT
TTTAT 3'

CE206 (SEQ ID NO:212): 5' AGCTATAAAAACTAGCTAGCTAGAAGCTTCTG
CAGGTCGACGTTAACTACGTACTAGCTAGCTAGTT
TTTCT 3'

Plasmid pJCA021 was obtained by inserting a 4900 bp PvuII-HindII fragment from pRW731-15 (previously described) into the SmaI and HindII sites of pBSSKT.

Th final insertion plasmid pRW874 had the three avian influenza HA genes transcribed in the same direction as the deleted F8 ORF. The left flanking arm of the plasmid adjacent to the H4 gene consisted of 2350 bp of fowlpox

sequenc . The right flanking arm adjacent to the H7 gene consisted of 1700 bp of fowlpox sequence. A linear representation of the plasmid is shown below.



Development of Recombinant vFP122. Plasmid pRW874 was transfected into TROVAC infected primary CEF cells by using the calcium phosphate precipitation method previously described (Panicali et al., 1982; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to specific H4, H5 and H7 radiolabelled probes and subjected to 5 sequential rounds of plaque purification until a pure population was achieved. Surface expression of all three glycoproteins was confirmed by plaque immunoscreen using specific reagents previously described. Stability of inserted genes was confirmed after two rounds of amplification and the recombinant was designated as vFP122.

Example 24 - COMPARISON OF THE LD_{50} OF ALVAC AND NYVAC WITH VARIOUS VACCINIA VIRUS STRAINS

Mice. Male outbred Swiss Webster mice were purchased from Taconic Farms (Germantown, NY) and maintained on mouse chow and water *ad libitum* until use at 3 weeks of age ("normal" mice). Newborn outbred Swiss Webster mice were of both sexes and were obtained following timed pregnancies performed by Taconic Farms. All newborn mice used were delivered within a two day period.

Viruses. ALVAC was derived by plaque purification of a canarypox virus population and was prepared in primary chick embryo fibroblast cells (CEF). Following purification by centrifugation over sucrose density gradients, ALVAC was enumerated for plaque forming units in CEF cells. The WR(L) variant of vaccinia virus was derived by selection of large plaque phenotypes of WR (Panicali et al., 1981). The Wyeth New York State Board of Health vaccine strain of vaccinia virus was obtained from Pharmaceuticals Calf Lymph Type vaccine Dryvax, control number 302001B. Copenhagen strain vaccinia virus VC-2 was obtained from Institut Merieux, France. Vaccinia virus strain NYVAC was derived from